

UNITED STATES PATENT APPLICATION

OF


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FOR


GENOMIC DNA FRAGMENTS CONTAINING REGULATORY AND CODING
SEQUENCES FOR THE $\beta 2$ -SUBUNIT OF THE NEURONAL NICOTINIC
ACETYLCHOLINE RECEPTOR AND TRANSGENIC ANIMALS MADE
USING THESE FRAGMENTS OR MUTATED FRAGMENTS

BACKGROUND OF THE INVENTION

Field of the Invention

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This invention relates to DNA and clones of $\beta 2$ -subunit of neuronal nicotinic acetylcholine receptor (nAChR) sequences. This invention also relates to genomic DNA fragments containing regulatory and coding sequences for the $\beta 2$ -subunit neuronal nAChR and transgenic animals made using these fragments or mutated fragments. The 5' flanking sequences contain a promoter, which confers neuron-specific expression. The genomic clones demonstrate the importance of the $\beta 2$ -subunit gene in the nicotinic system and in the pharmacological response to nicotine. The invention also relates to vectors containing the DNA sequences, cells transformed with the vectors, transgenic animals carrying the sequences, and cell lines derived from these transgenic animals. In addition, the invention describes the uses of all of the above.

References cited in this specification appear at the end by author and publication year or by cite number.

Neuron-specific expression. Many recombinant DNA-based procedures require tissue-specific expression. Unwanted or potentially harmful side-effects of gene transfer therapies and procedures can be reduced through correct tissue-specific expression. Furthermore, the ability to direct the expression of certain proteins to one cell type alone advances the ability of scientists to map, identify or purify these cells for important therapeutic or analytical purposes. Where the cells of interest

are neurons or a particular subset of neurons, a need for DNA sequences conferring neuron-specific or subset-specific expression exists.

Proteins expressed throughout an organism are often utilized for specific purposes by neurons. By expressing a particular subunit or component of these proteins solely in neuronal tissue, the neuron tailors the protein activity for its purposes. Finding the particular, neuron-specific subunits or components and unraveling why they are produced only in neuronal tissue holds the key to DNA elements conferring neuron-specific expression.

The inventors' knowledge of the biology of acetylcholine receptors provided an important foundation for this invention (see Changeux, The New Biologist, vol. 3, no.5, pp. 413-429). Different types of acetylcholine receptors are found in different tissues and respond to different agonists. One type, the nicotinic acetylcholine receptor (nAChR), responds to nicotine. A subgroup of that type is found only in neurons and is called the neuronal nAChR.

Generally, five subunits make up an acetylcholine receptor complex. The type of subunits in the receptor determines the specificity to agonists. It is the expression pattern of these subunits that controls the localization of particular acetylcholine receptor types to certain cell groups. The genetic mechanisms involved in the acquisition of these specific expression patterns could lead to an ability to control tissue-specific or even a more defined cell group-specific expression. The inventors' work indicates that defined elements in the

promoter sequence confer neuron specific expression for the $\beta 2$ -subunit.

The Pharmacological Effects of Nicotine. As noted above, nAChR responds to the agonist nicotine. Nicotine has been implicated in many aspects of behavior including learning and memory (1,2). The pharmacological and behavioral effects of nicotine involve the neuronal nAChRs. Studies using low doses of nicotine (23) or nicotinic agonists (16) suggest that high affinity nAChRs in the brain mediate the effects of nicotine on passive avoidance behavior. Model systems where neuronal nAChR has been altered can therefore provide useful information on the pharmacological effects of nicotine, the role of neuronal nAChR in cognitive processes, nicotine addiction, and dementias involving deficits in the nicotinic system.

Functional neuronal nAChRs are pentameric protein complexes containing at least one type of α -subunit and one type of β -subunit (3-5) (although the $\alpha 7$ -subunit can form functional homooligomers in vitro^{6,7}). The $\beta 2$ -subunit was selected for this study from among the 7 known α -subunits and 3 known β -subunits (3) because of its wide expression in the brain (8-10), and the absence of expression of other β -subunits in most brain regions (10). Mutation of this subunit should therefore result in significant deficits in the CNS nicotinic system. The inventors have examined the involvement of the $\beta 2$ -subunit in pharmacology and behavior. Gene targeting was used to mutate the $\beta 2$ -subunit in transgenic mice.

The inventors found that high affinity binding sites for nicotine are absent from the brains of mice homozygous for the $\beta 2$ -subunit mutation, $\beta 2$ -/- . Further, electrophysiological recording from brain slices reveals that thalamic neurons from these mice do not respond to nicotine application. Finally, behavioral tests demonstrate that nicotine no longer augments the performance of $\beta 2$ -/-mice on the test of passive avoidance, a measure of associative learning. Paradoxically, mutant mice are able to perform better than their non-mutant siblings on this task.

BRIEF SUMMARY OF THE INVENTION AND ITS UTILITY

In an aspect of this invention, we describe a 15 kb fragment of DNA carrying regulatory and coding regions for the $\beta 2$ -subunit of the neuronal nAChR. We characterize the promoter of the $\beta 2$ -subunit gene *in vitro* and in transgenic mice. We describe several DNA elements, including an E-box and other consensus protein-binding sequences involved in the positive regulation of this gene. Moreover, we show that the cell-specific transcription of the $\beta 2$ -subunit promoter involves at least two negative regulatory elements including one located in the transcribed sequence.

Preferred embodiments of these aspects relate to specific promoter sequences and their use in directing neuron-specific expression in various cells and organisms. An 1163 bp sequence and an 862 bp sequence both confer neuron-specific expression. Other embodiments include the -245 to -95 sequence of Figure 1,

containing an essential activator element, and the -245 to -824 sequence of Figure 1 containing a repressor. A repressor element composed of the NRSE/RE1 sequence is also present in the transcribed region. Certain plasmids comprising these genomic sequences are described as well.

The promoter sequences are important for their ability to direct protein, polypeptide or peptide expression in certain defined cells. For example, in the transgenic mice as shown below, proteins encoding toxins or the like can be directed to neurons to mimic the degradation of those cells in disease states. Others will be evident from the data described below.

Alternatively, the promoters can direct encoded growth factors or oncogenic, tumorigenic, or immortalizing proteins to certain neurons to mimic tumorigenesis. These cells can then be isolated and grown in culture. In another use, the promoter sequences can be operatively linked to reporter sequences in order to identify specific neurons *in situ* or isolate neurons through cell sorting techniques. The isolated, purified neurons can then be used for *in vitro* biochemical or genetic analysis. Reporter sequences such as LacZ and Luciferase are described below.

In another aspect of this invention, the inventors provide the genomic clones for mouse β -2 subunit of the neuronal nAChR. These clones are useful in the analysis of the mammalian nicotinic system and the pharmacology of nicotine. The inventors describe assays using transgenic mice where the genomic clones of the β 2-subunit have been used to knock out the high affinity binding of nicotine.

In addition to the deletion mutants described, mutations incorporated into the exons or regulatory sequences for the $\beta 2$ -subunit will result in useful mutant transgenic animals. These mutations can be point mutations, deletions or insertions that result in non-efficient activity of the nAChR or even a non-active receptor. With such mutant animals, methods for determining the ability of a compound to restore or modulate the nAChR activity or function are possible and can be devised. Modulation of function can be provided by either up-regulating or down-regulating receptor number, activity, or other compensating mechanisms. Also, methods to determine the ability of a compound to restore or modulate wild type behavior in the behavioral assays described or known (see 17, 22, 18, 2, 19, 21, 23, 24) can be devised with the mutant animals. Behavioral assays comprise, but are not limited to, testing of memory, learning, anxiety, locomotor activity, and attention as compared to the untreated animal or patient. Pharmacological assays (see 12, 13, 14, 15, 20) to select compounds that restore or modulate nAChR-related activity or behavior can thus be performed with the mutant animals provided by this invention. Dose and quantity of possible therapeutic agents will be determined by well-established techniques. (See, for example, reference 16.)

The present model systems comprising transgenic animals or cells derived from these animals can be used to analyze the role of nicotine on learning and behavior, the pharmacology of nicotine, nicotine addiction, and disease states involving deficits in the nicotinic system. In addition, potential

therapies for nicotine addiction or deficits in the nicotinic system can be tested with the transgenic animals or the cells and cell lines derived from them or any cell line transfected with a DNA fragment or the complete DNA of phage $\beta 2$ (CNCM accession number I-1503). These cell lines would include all those obtained directly from homozygous or heterozygous transgenic animals that carry or are mutated in the $\beta 2$ -subunit sequences. In addition, this would include cell lines created in culture using natural $\beta 2$ -subunit sequences or mutated $\beta 2$ -subunit sequences.

Techniques used could be, for example, those cited in PCT WO 90/11354.

Dementias, such as Alzheimer's disease, in which the high affinity nicotine binding site are diminished suggest that the present model can be used to screen drugs for compensation of this deficit. Accordingly, methods for screening compounds for the ability to restore or detectably effect activity of the neuronal nicotinic acetylcholine receptor comprising adding the compound to an appropriate cell line or introducing the compound into a transgenic animal can be devised. Transgenic animals and cell lines generated from this invention can be used in these methods. Such animal or cell line systems can also be used to select compounds which could be able to restore or to modulate the activity of the $\beta 2$ gene.

The transgenic animals obtained with the $\beta 2$ -subunit gene sequence (wildtype or mutated fragments thereof) can be used to generate double transgenic animals. For this purpose the $\beta 2$ -subunit transgenic animal can be mated with other transgenic

animals of the same species or with naturally occurring mutant animals of the same species. The resulting double transgenic animal, or cells derived from it, can be used in the same applications as the parent $\beta 2$ -subunit transgenic animal.

Both the promoter sequences and the genomic clones can be used to assay for the presence or absence of regulator proteins. The gel shift assays below exemplify such a use. The sequences or clones can also be used as probes by incorporating or linking markers such as radionuclides, fluorescent compounds, or cross-linking proteins or compounds such as avidin-biotin. These probes can be used to identify or assay proteins, nucleic acids or other compounds involved in neuron action or the acetylcholine receptor system.

Known methods to mutate or modify nucleic acid sequences can be used in conjunction with this invention to generate useful $\beta 2$ mutant animals, cell lines, or sequences. Such methods include, but are not limited to, point mutations, site-directed mutagenesis, deletion mutations, insertion mutations, mutations obtainable from homologous recombination, and mutations obtainable from chemical or radiation treatment of DNA or cells bearing the DNA. DNA sequencing is used to determine the mutation generated if desired or necessary. The mutant animals, cell lines or sequences are then used in the DNA sequences, systems, assays, methods or processes the inventors describe. The mutated DNA will, by definition, be different, or not identical to the genomic DNA. Mutant animals are also created by mating a first transgenic animal containing the sequences described here or made available

by this invention, with a second animal. The second animal can contain DNA that differs from the DNA contained in the first animal. In such a way, various lines of mutant animals can be created.

Furthermore, recombinant DNA techniques are available to mutate the DNA sequences described here, as above, link these DNA sequences to expression vectors, and express the $\beta 2$ -subunit protein or mutant derived from the $\beta 2$ -subunit sequences. The $\beta 2$ -subunit or mutant can thus be analyzed for biochemical or behavioral activity. In such a way, mutated DNA sequences can be generated that prevent the expression of an efficient nAChR.

Alternatively, the promoter sequences described can be used in expression vectors or systems to drive expression of other proteins. Obtainable DNA sequence can thus be linked to the promoter or regulatory sequences the inventors describe in order to transcribe those DNA sequences or produce protein, polypeptide, or peptides encoded by those DNA sequences.

DESCRIPTION OF THE RELATED ART

Previous studies by *in situ* hybridization (Wada et al., 1989; Hill et al., 1993; Zoli et al., 1994) and immunohistochemistry (Hill et al., 1993) demonstrate that all of the neuronal nAChR subunits cloned to date display a strict neuron-specific distribution. But different subunits exhibit an even tighter distribution to only small subsets of neurons in the brain. For example, the nAChR $\alpha 2$ -subunit transcripts are only detected in the *Spiriformis lateralis* nucleus in the chick diencephalon

(Daubas et al., 1990) or the *Interpeduncularis* nucleus in the rat (Wada et al., 1988). Also the $\beta 3$, $\beta 4$ and $\alpha 3$ -subunit transcripts are only detected in a small set of structures in vertebrate brain (references in Zoli et al., 1994).

The nAChR, $\alpha 4$, $\alpha 5$, $\alpha 7$, and $\beta 2$ -subunit gene transcripts, in comparison, show a much wider distribution. (Wada et al., 1989; references in Zoli et al., 1994). For example, the $\beta 2$ -subunit transcripts are found in the majority of neurons in the CNS and in all the peripheral neurons that express the nAChR (Role, 1992; Hill et al., 1993).

As a consequence of the differential expression of these subunits, a wide diversity of nAChR species occurs in vertebrates. Each species has a defined pattern of expression involving diverse categories or groups of neurons. For example, the neurons from medial Habenula interconnect with those from the *Interpeduncularis* nucleus and yet each express distinct sets of nAChR subunits (see Role, 1992 for review) exhibiting different physiological and pharmacological profiles (Mulle et al., 1991).

Only limited information is available, to date, about the genetic mechanisms that account for regulation of nAChR gene transcription in neurons. Previous work on the promoter of the chick $\alpha 7$ subunit gene analyzed *in vitro* failed to characterize the DNA elements responsible for transcriptional regulation (Matter-Sadzinski et al., 1992). In another study, the promoter of the $\alpha 2$ -subunit gene was partially characterized and a silencer described and sequenced (Bessis et al., 1993, see also Daubas et al. 1993).

Certain evidence leads to the study of the $\beta 2$ -subunit in particular. It is expressed in the majority of the neurons in the brain (Hill et al., 1993). Also, the timing of the appearance of the $\beta 2$ -transcripts closely parallels that of neuronal differentiation (Zoli et al., 1994). We thus decided to study the genetic mechanisms that regulate its transcription.

BRIEF DESCRIPTION OF THE INVENTION

Gene Structure

We have cloned a genomic fragment containing the regulatory sequences and sequences encoding the mouse nAChR $\beta 2$ -subunit gene. The inventors have found that at least part of the regulatory region is conserved among different mammalian species. Particularly, the region between +16 to +38 bp corresponding to the NRSE/RE1 as described in Figure 1. Using RNase protection and amplification of primer extension products, we found one main and three minor transcription start sites (Figure 1). The primer extension experiments were performed using two different reverse transcriptases, with different batches of mRNA and with different primers. These PCR based techniques allowed us to amplify and subclone the same fragments corresponding to transcription start sites rather than reverse transcriptase stops. The transcription start sites that we have characterized are located downstream from the position of the longest rat (Deneris et al., 1988) and human (Anand and Lindstrom, 1990) $\beta 2$ cDNA 5' end (see Figure 1). This implies that in human and rat, another transcription start site is used. Such a discrepancy between species has already been

demonstrated for the ϵ -subunit of the muscle nAChR (Dürr et al., 1994, see also Dong et al., 1993; Toussaint et al., 1994). In contrast with the $\alpha 2$ subunit gene (Bessis et al., 1993), no upstream exon could be detected.

Structural analysis of a 1.2 kbp flanking region disclosed many consensus motifs for nuclear protein binding including an Sp1 site and an E-box. Approximately 90 bp of the undeleted 1.2 kb promoter are transcribed and this region contains a NRSE/RE1 sequence (Kraner et al., 1992; Mori et al., 1992). Regulatory elements have already been described downstream of the transcription start site in different systems such as the Polyomavirus (Bourachot et al., 1989) or the fos gene (Lamb et al., 1990).

B The promoter region is located between the Eco47III located in exon 1 (see Figure 1) and the BamHI site 4.5 kb upstream. One preferred embodiment is the 1163 bp sequence described in Figure 1 between the EcoRI and Eco47III sites. Regulatory sequences may be located in the 2 kb downstream from the Eco47III site. The regulatory elements from the nAChR $\beta 2$ -subunit sequences can be used to direct the neuron specific expression of a nucleotide sequence encoding a protein, polypeptide or peptide linked to them. Said protein, polypeptide, or peptide can be toxins, trophic factors, neuropeptides, tumorigenic, oncogenic, or immortalizing proteins, or any other protein that can change the function of the neuron.

A 1163 bp Promoter Achieves Cell-Specific Transcription.

The 1163 bp promoter contains regulatory sequences for both tissue-specific and temporal specific transcription of the $\beta 2$ -subunit gene. Transient transfection experiments showed that the 1163 bp fragment contains sufficient information to confer cell-specific expression of the nAChR $\beta 2$ -subunit gene. We showed that the same promoter directs a strict cell-specific transcription of the β -galactosidase (β -gal) reporter gene. Moreover, the transgenic construct appears to be activated with the same timing as the endogenous $\beta 2$ -subunit gene during the development of the early embryonic nervous system (Zoli et al., 1994). At later stages of development, most of the peripheral $\beta 2$ expressing neurons are still labelled (Figure 4C, D).

The promoter sequence was tested in transgenic mice by generating two lines (13 and 26) expressing β -gal under the control of the $\beta 2$ -subunit promoter. In CNS, the pattern of β -galactosidase expression is different between the two lines. Only a subset of the cells that normally express $\beta 2$ express the transgene. This type of discrepancy between the expression of the transgene and the endogenous gene has already been described for the dopamine β -hydroxylase gene promoter (Mercer et al., 1991; Hoyle et al., 1994) or for the GAP-43 gene (Vanselow et al., 1994). Unexpected expression has been observed in transgenic line 13 in the genital tubercle and in skin muscles. This expression is likely to be due to the integration site of the transgene as these tissues are not stained in line 26. To our knowledge, most of the neuronal promoters studied by transgenesis display ectopic

expression in a certain small percentage of transgenic lines (Forss-Petter et al., 1990; Kaneda et al., 1991; Banerjee et al., 1992; Hoesche et al., 1993; Logan et al., 1993, Vanselow et al., 1994). However, techniques in the art afford the construction of lines where the expression pattern of the transgene closely mirrors or duplicates that of the original gene. See references for further details showing the success of the transgenesis procedure.

By comparing the β -gal positive cell distribution with those of other known neuronal markers, it becomes apparent that a similarity exists with the distribution of choline acetyltransferase, TrkA (the high affinity nerve growth factor receptor) and p^{75} (the low affinity nerve growth factor receptor) expressing cells (Yan and Johnson, 1988; Pioro and Cuello, 1990a, b; Ringstedt et al., 1993). In particular, in developing rats, p^{75} is expressed in almost all the peripheral ganglia and central nuclei (with the exception of the zona incerta and hypothalamic nuclei), which express the transgene (Yan and Johnson, 1988). It is also interesting to note that p^{75} expression (like the expression of the $\beta 2$ -promoter transgene) is transient in many peripheral ganglia and brain nuclei, decreasing to undetectable levels at perinatal or early postnatal ages. It is therefore possible that the $\beta 2$ -subunit promoter contains an element controlled by the activation of p^{75} , or that both the $\beta 2$ transgene and p^{75} gene are controlled by a common regulator.

In conclusion, although the promoter seems to lack some regulatory elements active in the brain, the existing regulatory

elements are sufficient to allow a cell- and development-specific expression of β -galactosidase in the PNS, in the spinal cord, and in several brain structures. The promoter can also be used in assays to identify regulator proteins in neuronal tissue.

DNA Regulatory Elements.

To further characterize the DNA elements involved in the transcription of the $\beta 2$ subunit gene, we deleted or mutated the 1163 bp promoter and analyzed the resulting constructs by transient transfection. A repressor element present in the distal 5' end region is active in fibroblasts but not in neuroblastomas. This element thus accounts, at least in part, for the neuron-specific expression of the $\beta 2$ -subunit gene. Further analysis of the promoter shows that deleting 589 bp increases the activity in neuroblastomas, but not in fibroblasts (Figure 6, compare 862E and 283E-Luci).

An NRSE/RE1 element is located at the 3' extremity of the promoter. This element has already been shown to restrict the activity of promoters in neuronal cells (Kraner et al., 1992; Mori et al., 1992; Li et al., 1993). In the 1163 bp promoter of the $\beta 2$ -subunit gene, point mutation of this sequence leads to a ~100 fold increase of the transcriptional activity in fibroblasts implying that this sequence is involved in the neuron-specific expression of the $\beta 2$ -subunit gene. Moreover, sequence comparison shows that this sequence is highly conserved in rat and human $\beta 2$ -subunit cDNAs (Deneris et al., 1988; Anand and Lindstrom, 1990) as well as in several promoters of genes expressed in the nervous system, such as the middle-weight neurofilament gene, the

CAM-L1 gene, the Calbinbin gene, or the cerebellar Ca-binding protein gene (see Table 1B).

Deletion experiments described in Figure 6 show that an essential activator element is present between nucleotides -245 and -95. An Sp1 binding site and an E-box could be detected in this region. Sp1 sites are ubiquitous factors, whereas E-boxes have been involved in several genetic regulatory mechanisms in muscle (see Bessereau et al., 1994 for the nAChR α 1-subunit) as well as in neurons (Guillemot et al., 1993). Dyad elements have also been reported in some neuronal promoters, such as those of the Tyrosine hydroxylase gene (Yoon and Chikaraishi, 1994), the SCG10 gene (Mori et al., 1990), the GAP43 gene (Nedivi et al., 1992), or in the flanking region of the N-CAM gene (Chen et al., 1990). Results shown in Table 1A demonstrate that in neuroblastomas, the 1163 bp promoter mutated in the E-box/Dyad is significantly less active than the wild type promoter. Moreover, a gel shift assay (Figure 7) further demonstrates that the E-box/Dyad is able to bind specific complexes. This suggests that the E-Box/Dyad is responsible for at least part of the activation of β 2-subunit gene transcription. However, transactivation experiments of heterologous promoters suggest that the E-box cooperates with the Sp1 site located 27 bp upstream to positively activate transcription. This type of cooperation between an E-Box and an Sp1 binding site has already been demonstrated for the regulation of the muscle nAChR α 1-subunit transcription (Bessereau et al., 1993).

In conclusion, we have shown that the $\beta 2$ -subunit gene is primarily regulated by negatively acting elements and by one positive element that comprises an E-box. This double regulation seems to be a general feature shared by several neuronal genes (Mandel and Mckinnon, 1993) and allows fine tuning of the transcription of neuronal genes. Moreover, our transgenic studies show that the 1163 bp promoter confers a tight neuron-specific expression, but lacks some developmental or CNS-specific regulatory elements.

DESCRIPTION OF THE FIGURES

Figure 1: Nucleotide sequence of the region surrounding the initiator ATG of the $\beta 2$ -subunit gene.

The four vertical arrowheads show the four extremities found using RACE-PCR and SLIC, corresponding to the transcription start sites. The vertical arrows indicate the position corresponding to the 5' end of the longest rat (r) and human (h) $\beta 2$ -subunit cDNA clones (Deneris et al., 1988). The endpoints of the deletions used in the experiments described in Figure 3 are indicated above the sequence. Nucleotides located in the intron are typed in lower cases.

Figure 2: Mapping of the 5' end of the $\beta 2$ -subunit mRNA.

A. RNase protection experiments. Total RNA from DBA2 mouse brain (5 and 15 μ g, lane 2 and 3 respectively) and yeast tRNA (15 μ g, lane 1) were hybridized to a 32 P-labeled RNA probe containing 158 nucleotides of intron 1, and 789 nucleotides of upstream sequences (-634/+155). The size of the protected bands

were estimated according to the lower mobility in acrylamide of RNA as compared to DNA (Ausubel et al., 1994) and by comparison with the sequence of M13mp18 primed with the universal primer. The arrow on the left part of the gel points to the major protected band.

B. Identification of the transcription start site using SLIC. The lower part of the Figure shows the strategy and describes the oligonucleotides used for the SLIC or the RACE-PCR. In the SLIC experiment, a primer extension was performed using oligonucleotide pEx3. The first strand of the cDNA was subsequently ligated to oligonucleotides A5', and the resulting fragment was amplified using oligonucleotides A5'-1/p0 then A5'-2/p1. The amplified fragment was then loaded onto a 1.2% agarose gel. The gel was blotted and hybridized to oligonucleotide p2. Lane 1 : 5 μ g of total DBA2 mouse brain RNA. Lane 2-3 : controls respectively without reverse transcriptase and without RNA. Minus: the T4 RNA Polymerase was omitted. Same result was obtained using RACE-PCR.

Figure 3: Cell-specific expression of the β 2-subunit promoter in vitro.

The luciferase activity of the plasmids were normalized to the activity of the promoterless plasmid (KS-Luci, described in Materials and Methods). RACE-PCR on mRNA extracted from SK-N-Be transfected with EE1.2-Luci, using luciferase oligonucleotides (described in Material and Methods) showed that the amplified fragment had the expected size for the correct transcription initiation site.

Figure 4: Cell-specific expression of the $\beta 2$ -subunit promoter in transgenic mice.

A. Whole mount coloration of E13 embryos. The arrowheads point to ectopic expression in skin muscles. B. Detection of the β -galactosidase activity in a parasagittal section of an E13 embryo at the lumbo-sacral level. Arrowheads indicate labelling in the ventral and dorsal horn of the spinal cord. C. Detection of the $\beta 2$ -subunit transcripts in an adjacent section of the same embryo. dr: dorsal root ganglion; t : tectum; og : orthosympathetic ganglionic chain; tr: trigeminal ganglion.

Figure 5: Expression of β -galactosidase in transgenic mice.

A. staining of the retina(re) and the trigeminal ganglia (tr) (E14.5). B. staining of cardiac parasympathetic ganglionic neurons (pg) (E14.5). C. transverse section of the spinal cord (P1). dr: dorsal root ganglion, og: orthosympathetic ganglion. D. Ventral view of the spinal cord (P1). The smaller arrows indicate neurons that have not been identified.

Figure 6: Expression of the Luciferase fusion genes containing 5' end deletions of the β -subunit promoter.

Plasmids are called nnnE-Luci, where nnn is the size in nucleotides of the insertion, and E is the 5' end restriction site (Eco47III). The arrow indicates the transcription start site. The activities of EE1.2-Luci are from Figure 3.

Figure 7: Gel shift experiment. Autoradiogram of the mobility shift experiment. The probe used was a ^{32}P labelled double stranded E-D oligonucleotide. This oligonucleotide carries only the E-Box/Dyad element, whereas the oligonucleotide S-E

carries the Sp1 binding site as well as the E-Box/Dyad element. The competitor oligonucleotides were used in 10- and 100-fold molar excess, except for S-E that was used only in 100-fold molar excess.

Table 1: Positive and negative regulatory elements in the proximal region of the 1163 bp promoter.

A. Effect of mutations in the proximal part of the 1163 bp promoter. The activities of the wild type or mutated promoters are normalized to the luciferase activity of the promoterless KS-Luci plasmid. The activities of EE1.2-Luci are from Figure 3.

B. Alignment of the proximal silencer of the β -subunit promoter with other neuronal promoters. The sequences are taken from (Maue et al., 1990, Na channel, accession number M31433), (Mori et al., 1990; SCG10, M90489), (Sauerwald et al., 1990; Synapsin I, M55301), (Kohl et al., 1992; CAML1 gene, X63509), (Gill and Christakos, 1993, Calbindin gene, L11891), (Zopf et al., 1990; Neurofilament gene, X17102, reverse orientation). The numbering refers to the sequences in the GenBank/EMBL library.

Figure 8: Disruption of the gene encoding the β 2-subunit of the neuronal nAChR. a-i, Normal genomic structure of the mouse β 2-subunit gene. Portion of exon one removed by the recombination event is shaded in light grey. ATG - initiator methionine. Boxes represent exons I-IV. a-ii, Targeting replacement vector used to disrupt the endogenous β 2-subunit gene. Initiator methionine and the rest of the first exon were replaced with the coding region of NLS-lacZ and the MCl neo^R expression cassette²⁵. The construct was able to direct lacZ

expression after stable transfection of PC12 cells (not shown), but lacZ expression was never detected in recombinant animals, despite the lack of obvious recombination in the lacZ DNA. Diphtheria toxin-A gene (DTA)²⁶ was used to select against random integration. a-iii, Structure of the mutated $\beta 2$ -gene.

Restriction sites: H, HindIII; R, EcoRI; E, Eco47III; P, PstI. Black arrows, primers used to detect recombination events in embryonic stem (ES) cells. Grey arrows, primers used to detect the wildtype or mutated $\beta 2$ genes. b, PCR analysis of tail DNA from a +/+, +/- and a -/- mouse. c, Southern blot analysis of tail DNA restricted with HindIII from the same mice analyzed in panel b. d, Western blot analysis of total brain protein using a monoclonal antibody raised against the $\beta 2$ -subunit.

METHODS: a, The $\beta 2$ -targeting vector was constructed by inserting a multiple cloning site (MCS) into the Mcl neo cassette (GTC GAC GGT ACC GCC CGG GCA GGC CTG CTA GCT TAA TTA AGC GGC CGC CTC GAG GGG CCC ATG CAT GGA TCC). A 4.1 kb EcoRI-Eco47III $\beta 2$ -genomic fragment 5' to the ATG and a 1.5 kb PstI $\beta 2$ -genomic fragment starting within the first intron of the $\beta 2$ -gene were cloned into the MCS. Hm1^{27, 28} embryonic stem cells (5×10^7) were transfected with the linearized targeting vector by electroporation as described²⁵. Twenty-four surviving

G418-resistant clones were screened by PCR ($\beta 2$ -primer - GCC CAG ACA TAG GTC ACA TGA TGG T; neo-primer - GTT TAT TGC AGC TTA TAA TGG TTA CA). Four were positive and were later confirmed by

Southern blot analysis. Clones were injected into 3.5-day-old blastocysts from non-agouti, C57BL/6 mice and planted in receptive

females. All resulting male chimaeric mice were mated to F1, C57BL/6xDBA/2 non-agouti females. Of 15 chimaeras, one showed germ-line transmission. $\beta 2$ +/- heterozygotes were mated and offspring were evaluated by PCR analysis (panel b). b, PCR was 35 cycles of 94°/1 min, 65°/2 min and 72°/1 min. c, Southern blotting was performed as described²⁹. The 1.5 kB PstI genomic fragment used for the targeting construct was labelled by random priming. d, Western blotting was performed as described²⁹ using monoclonal antibody 270¹¹.

Figure 9: Mapping of the neuronal nAChR in mouse brain using *in situ* hybridization and tritiated nicotine binding. A, *In situ* hybridization using antisense oligonucleotide probes based on the sequence of the cDNAs encoding the $\beta 2$ -, $\alpha 4$ - and $\beta 4$ -subunits of the nAChR to detect their respective mRNAs in serial sections from the brains of $\beta 2$ +/+, +/- and -/- mice. Midthalamic sections are shown. White arrows indicate the MHb labelled by the $\beta 4$ -antisense oligonucleotide. B, Receptor autoradiography using tritiated nicotine revealing high affinity binding sites in the brains of wildtype, heterozygous and $\beta 2$ -mutant mice. Representative sections at the level of the striatum, thalamus and tectum are shown.

METHODS, A, *In situ* hybridization was performed as follows:

In situ hybridization procedure. Frozen tissues were cut at the cryostat (14 μ m thick sections), thaw mounted on poly-L-lysine coated slides and stored at -80 C for 1-3 days. The procedure was carried out according to Young et al. (1986). Briefly, sections were fixed with 4% paraformaldehyde for 5 min.

at room temperature, washed in phosphate buffered saline (PBS) and then acetylated and delipidated in ethanol and chloroform (5 min.). They were prehybridized for 2-4 h at 37 C under parafilm coverslips. The composition of the prehybridization and hybridization mixtures was 50% formamide, 0.6M NaCl, 0.1M dithiothreitol, 10% dextran sulfate, 1mM ethylenediaminetetraacetic acid (EDTA), 1XDenhardt's solution (50x = 1% bovine serum albumin/1% Ficoll/1% polyvinylpyrrolidone), 0.1mg/ml polyA (Boehringer), 0.5mg/ml yeast RNA (Sigma), 0.05 mg/ml herring sperm DNA (Promega) in 0.02M Tris-HCl, pH 7.5. Probes were applied at a concentration of 2000-3000 Bcq/30 μ l section (corresponding to around 15 fmol/section). After removal of coverslips and initial rinse in 2X standard saline citrate (SSC) solution (3M NaCl/0.3M sodium citrate) at room temperature (two time for 5 min.), sections were washed four times for 15 min in 2X SSC/50% formamide at 42°C and, then, two times for 30 min in 1X SSC at room temperature. 1 mM dithiothreitol was added to all washing solutions. After rinsing in ice-cold distilled water and drying, they were exposed for 10-20 days to Hyperfilm β max (Amersham) and then to a photographic emulsion (NTB2, Kodak) for 1-2 months.

Analysis of histological preparations. The analysis of the labelling pattern for the different mRNAs was carried out both on film and emulsion autoradiograms. Identification of anatomical structures was carried out after counterstaining of the serial sections of the entire embryos with toluidine blue. Definition of anatomical areas in the brain and recognition of peripheral

nervous system (PNS) structures was based on different atlases, including The Rat Brain in Stereotaxic Coordinates (Paxinos and Watson, 1986), the Atlas of Developing Rat Brain (Paxinos et al. 1991), the Atlas of Mouse Development (Kaufman, 1992), and the Atlas of the Prenatal Mouse Brain (Schambra et al., 1992). For cranial nerve ganglia development, the plates and descriptions from Altman and Bayer (1982) were consulted. In order to confirm the identification of some central and peripheral structures (e.g., cranial nerve motor nuclei, autonomic motor ganglia) in situ hybridization for choline acetyltransferase was performed on some sections.

A score from 1 + (low intensity) to 3+ (high intensity) was assigned to the labelling of the anatomical structures based on the subjective evaluation of two experimenters. Background labelling was considered the density of grains in nonneural tissues high cellularity (such as the liver and muscles) or with high density of extracellular matrix (such as cartilage) or the density of labelling over neural structures after displacement with 20X cold probe. In the absence of grain counting at the cellular level, the scores must be regarded with caution. For instance, decreases in labelling intensity of a developing structure may be due to dispersion of positive cells in the structure caused by multiplication of negative cells or formation of neuronal processes. Though the oligonucleotides had the same length and they were labelled according to the same protocol, no attempt to compare the signal intensity or different transcripts was made. Unless specified otherwise, the labeling shown in the

pictures has been obtained by using oligonucleotides no. 31 ($\alpha 3$), 47 ($\alpha 4$), 51 ($\alpha 2$), and 62 ($\alpha 4$) (see Table 1 for oligonucleotide characteristics).

Specificity controls. For each mRNA, two to four oligonucleotides were selected in unique parts of the sequence (e.g., the putative cytoplasmic loop between M3 and M4 for nAChR subunits). An initial assessment of the specificity was performed by searching for possible homology with other known sequences in Genbank/EMBL. As histological tests for specificity were considered the following: 1. Two or more oligonucleotide probes for each mRNA gave the same hybridization pattern (fig. 1). 2. The pattern of labelling in central structures in the adult rat was in agreement with that observed by other authors (Wada et al., 1989; Dineley-Miller and Patrick, 1992). 3. Given that most oligonucleotides used were 45-mers with similar GC content (Table 1), each oligonucleotide probe constituted a control for the specificity of the others. 4. The addition to the hybridization mixture of a 20-fold excess of cold probe produced a complete disappearance of the labelling (Fig. 2).

The oligonucleotide probes used fulfilled all these criteria, with the exception of the four probes against $\alpha 3$ mRNA, which did not satisfy criterion 2. Previous studies based on cRNA probes showed a relatively widespread distribution of this subunit mRNA in adult rats, notably high levels in the cerebral cortex layer IV, entorhinal cortex layer II, anterior and ventral thalamic nuclei, medial and lateral geniculate nuclei, medial habenula, posterior hypothalamus and supramammillary nuclei, pineal gland,

motor nuclei of the V and VII nerves, locus coeruleus, nucleus ambiguus, and area postrema (Wada et al. 1989). At variance with these observations, in adult rats we could detect high levels of $\alpha 3$ mRNA signal only in the medial habenula, intermediate in the pineal gland, area postrema, motor nucleus of the V nerve and cerebellum, low in a few thalamic nuclei and locus coeruleus. Part of the discrepancy may be ascribed to a lower sensitivity of oligonucleotide probes versus riboprobes. However, considering the difficulty of carrying out specificity controls for cRNA probes, especially when hydrolysis of the probe is performed in the histological procedure (Wada et al., 1989), it is possible that some labelling previously attributed to $\alpha 3$ mRNA actually derives from hybridization to other (nAChR-related) RNA sequences.

B Oligonucleotides: $\beta 2$: 5'-TCG CAT GTG GTC CGC AAT GAA GCG TAC GCC
 B ATC CAC TGC TTC CCG-3'; $\alpha 4$: 5'-CCT TCT CAA CCT CTG ATG TCT TCA
 B AGT CAG GGA CCT CAA GGG GGG-3'; $\beta 4$: 5'-ACC AGG CTG ACT TCA AGA CCG
 GGA CGC TTC ATG AAG AGG AAG GTG-3'. B, ^3H -nicotine binding was

performed as described by Clarke et al.³⁰. Fourteen μm coronal sections were incubated at room temperature for 30 min. in 50 mM Tris pH 7.4/8 mM CaCl_2 /4 nM ^3H -L-nicotine. Nonspecific binding was evaluated in the presence of 10 μM L-nicotine bitartrate. Following incubation, sections were rinsed 2 x 2 min. in ice cold PBS and briefly rinsed in ice cold water. Slides were exposed for 60 days to Hyperfilm ^3H .

Figure 10: Patch clamp recording of nicotine evoked currents in the MHb and anterior thalamus of $\beta 2$ +/+ and -/- mice. A, Representative recordings from cells in the MHb and the anterior

thalamus of wildtype and $\beta 2$ $-/-$ mice. The off-rate of the agonist is significantly greater in the MHb than in the anterior thalamus, resulting in a different kinetics of response in the two structures. The response to nicotinic agonists of the MHb is maintained in $\beta 2$ $-/-$ animals, while the response to nicotinic agonists of the anterior thalamus is completely abolished in $\beta 2$ $-/-$ mice. B, table of responses to nicotinic agonists in various nuclei of $\beta 2$ $+/+$ and $-/-$ mice.

METHODS, Coronal slices were obtained from the thalamus of 8-12 day old mice using a Dosaka slicer in ice cold ACSF medium (125 mM NaCl/26 mM NaHCO_3 /25 mM Glucose/1.25 mM NaH_2PO_4 /2.5 mM KCl 2.5/2 mM CaCl_2 /1 mM MgCl_2 pH 7.3). Slices were maintained in the same medium for 1-8 hours. Cells in slices were visualized through a Zeiss microscope. Whole cell recordings were obtained with 2-4 MOhm hard-glass pipettes containing 150 mM CsCl/10 mM EGTA/10 mM HEPES/4 mM di-sodium ATP/4 mM MgCl_2 pH adjusted to 7.3 with KOH. Five to ten sec. pulses of drug were applied rapidly to the cell through a 50 μM diameter pipette above the slice, fed by gravity with a solution containing 150 mM NaCl/10 mM Hepes/2.5 mM KCl/2 mM CaCl_2 /1 mM MgCl_2 . Recordings were made in the presence of CNQX (5 μM) and of the GABA_A antagonist SR-95531 (10 μM). Currents were recorded with an Axopatch ID (Axon Instrument) patch amplifier, digitized on a Compaq PC and further analyzed with the PClamp program (Axon Instrument).

Figure 11: Performance of $\beta 2$ $-/-$ mice and their wildtype siblings on the passive avoidance test. A, response to various levels of footshock in retention test following a post-training

injection of either vehicle or nicotine (10 μ g/kg). Average step-through latency during the training trial was 17.0 +/- 3.6 sec for mutant mice and 15.0 +/- 3.5 sec for their nonmutant siblings. B, bar graph showing the difference in retention latency between wildtype and homozygous β 2 mutant mice injected with either vehicle or nicotine (10 μ g/kg) at foot shock intensity of 2.00 mA. Data are represented as means +/- S.E.M. of the following groups: wildtype + vehicle (n=27); wildtype + nicotine (n=23); β 2-mutant mice + vehicle (n=17); β 2-mutant mice + nicotine (n=17). Statistical analysis was performed using a mixed factorial analysis of variance followed by a-posteriori testing of simple effects. #, p < 0.05, wildtype vs mutant mice following vehicle injection; *, p < 0.01, nicotine vs vehicle in wildtype mice.

METHODS, Passive avoidance test was performed as described in the text, according to Nordberg and Bergh²⁰ and Faiman et al²⁰. Nicotine (bitartrate, Sigma) was freshly dissolved in PBS. IP injection of the same volume of either nicotine or vehicle immediately followed footshock during the training trial.

Figure 12: Phage and plasmids containing all or part of the β 2-subunit gene and the promoter. In the names of the plasmids, the numerals indicate the size of the fragment and the letters indicate the restriction sites used to generate it.

DETAILED DESCRIPTION

The descriptions and examples below are exemplary of the embodiments and scope of this invention. The invention is not

limited to the scope of this description. Furthermore, this description together with the accompanying sections of this specification and the material incorporated by reference enables the practice of all of the claims which follow.

The examples and embodiments that follow of course can be modified by techniques known in the art. Variations in the nucleic acid sequences described or claimed can be produced by known methods without altering the effects or advantages the inventors have shown. Such variations are therefore included within the scope of this description and invention.

Materials and Methods

Isolation of Genomic Clones.

The PCX49 plasmid (Deneris et al., 1988) containing the entire rat cDNA (kindly provided by Drs. J. Boulter and S. Heinemann, The Salk Institute, San Diego, CA) was cut with EcoRI, the ~2.2 kb fragment was isolated and used as a probe to screen an EMBL3 bacteriophage library of mouse DBA2 genomic DNA. One unique clone was obtained spanning ~15 kb of DNA upstream and ~5 kb downstream from the first exon. Figure 1 shows the nucleotide sequence of 1.2 kb upstream from the initiator ATG.

Hybridization conditions can be modified by known techniques²⁹ to determine stringent conditions for this probe. Changes in the hybridization conditions such as temperature (from about 45°C to about 65°C) and SSC buffer concentration (from about 0.1xSSC to about 6xSSC), as well as changes in the temperature of and the buffer for the washing conditions can be made to develop

sufficiently stringent conditions that allow hybridization to the $\beta 2$ -subunit sequences. Other related sequences can thus be isolated from other libraries based on this hybridization procedure. Human sequences will be isolated by using hybridization conditions such as 45°C and 6xSSC.

Three deposits were made on December 13, 1994 at the Collection Nationale de Cultures de Microorganismes (CNCM), Institut Pasteur, 25 Rue du Docteur Roux, 75724 PARIS CEDEX 15, France. A phage, $\lambda\beta 2$ nAchR, is deposited under the accession number I-1503. This phage contains 15-20 kb of genomic DNA including the promoter sequences and the coding sequences for all of the exons of the murine $\beta 2$ -subunit of neuronal nAchR. Two *E.coli* cultures bearing plasmids have also been deposited. Plasmid pSA9 in *E. coli* DH5 α has accession number I-1501 and contains 9 kb of murine genomic DNA including the regulatory sequences and regions coding for exons 1, 2 and 3 of the $\beta 2$ -subunit. Plasmid pEA5 in *E. coli* DH5 α has accession number I-1502 and contains 5 kb of murine genomic DNA including a region of about 1.2 kb upstream of the Eco47-III site and a region coding for exons 1 to 5 of the $\beta 2$ -subunit. The inventors intend to deposit the nucleotide sequence data reported here in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number: X82655.

Mapping of the Transcription Initiation Site.

For the mRNA mapping, we used different batches of total RNA extracted from DBA2 embryos at stage E13 or E15. The RNA samples were first digested with DNase I to avoid DNA contamination.

RNase protection. An XbaI/PstI fragment containing part of intron 1 was inserted into Bluescript SK (Stratagene). The plasmid was then linearized by BglII, and an RNA probe was synthesized using the T7 promoter. The protection experiments were then performed as described in Ausubel et al. (1994).

RACE-PCR (Frohman et al., 1988). The mRNA was hybridized 5 minutes at 80°C with 10 pmol of primer. The synthesis of the cDNA was performed using 400u MMLV (Gibco) for 45 minutes at 37°C in the buffer recommended by the supplier. After a phenol/chloroform extraction, the cDNA was ethanol precipitated. The terminal transferase reaction was performed in 0.2 M potassium cacodylate; 25 mM Tris-HCl pH 6.6; 25 mg/ml BSA; 1.5 mM CoCl₂; 50 nM dATP and 50u Terminal transferase (Boehringer) for 30 minutes at 37°C. After phenol/chloroform extraction and ethanol precipitation, one tenth of the terminal transferase reaction was amplified using Promega's Taq DNA polymerase (30 cycles, 1 minute at: 94°C; 55°C; 72°C). The amplified fragment was then loaded on an agarose gel. The gel was blotted and hybridized to oligonucleotide p2. We used pEx2 as a primer for cDNA synthesis, and p0/BEpT for PCR to map mRNA from brain. OLUCI3 (synthesis of cDNA) and OLUCI2/BEpT (PCR) were used to map mRNA from transfected cells.

SLIC (Dumas Milnes Edwards et al., 1991). The cDNA was first synthesized from 5 µg total RNA using pEx3 (6 pmol) as a primer in 50 mM Tris-HCl pH 8.3; 8 mM KCl; 1.6 mM MgCl₂; 5 mM spermidine; 0.5 mM dNTP; 1u/µl RNasin; 0.1 mg/ml BSA; 70 mM β-mercaptoethanol; 80u AMV reverse transcriptase (Promega) at 42°

for 45 minutes. The RNA was subsequently degraded in NaOH. The first strand of the cDNA was then ligated with the oligonucleotide A5'. The resulting single stranded cDNA was then submitted to two rounds of PCR amplification with oligonucleotides A5'-1/p0 and A5'-2/p1 (35 cycles 94°C 1 minute; 60°C 30 seconds; 72°C 45 seconds).

The sequence of the oligonucleotides were the following:

(SEQ ID NO: 4)
 B A5' : 5'-CTGCATCTATCTAATGCTCCTCTCGCTACCTGCTCACTCTGCGTGACATC
 (SEQ ID NO: 5)
 B A5'-1 : 5'-GATGTCACGCAGAGTGAGCAGGTAG
 (SEQ ID NO: 6)
 B A5'-2 : 5'-AGAGTGAGCAGGTAGCGAGAGGAG
 (SEQ ID NO: 7)
 B p0 : 5'-CCAAAGCTGAACAGCAGCGCCATAG
 (SEQ ID NO: 8)
 B p1 : 5'-AGCAGCGCCATAGAGTTGGAGCACC
 (SEQ ID NO: 9)
 B p2 : 5'-AGGCGGCTGCGCGGCTTCAGCACCACGGAC
 (SEQ ID NO: 10)
 B pEx2 : 5'-GCCGCTCCTCTGTGTCACTACCCAAAACCC
 (SEQ ID NO: 11)
 B pEx3 : 5'-ACATTGGTGGTCATGATCTG
 (SEQ ID NO: 12)
 B BEpP : 5'-GCGGGATCCGAATTC(T)₂₁ A/C/G
 (SEQ ID NO: 13)
 B OLUCI3 : 5'-CGAAGTATTCCGCGTACGTGATG
 (SEQ ID NO: 14)
 B OLUCI2 : 5'-ACCAGGGCGTATCTCTTCATAGC

Construction of Plasmids.

KS-Luci: The HindIII/KpnI restriction fragment of the pSVOAL plasmid (de Wet et al., 1987) was subcloned in the corresponding site of Bluescript KS. The most 5' EcoRI/BsmI (45 bp) fragment of the Luciferase gene was then deleted according to (de Wet et al., 1987) and replaced by a Sal I site. The 342 bp PvuII/HindIII restriction fragment of SV40 containing the

polyadenylation sites was subsequently subcloned into the EagI sites using adaptors.

EE1.2-Luci: The 1.2 kbp EcoRI/Eco47II fragment of the λ 2 phage was inserted in the EagI/SaII sites of KS-Luci using adaptors. The 5' end deletions of the promoter were obtained using Bal3.1 exonuclease as in Current Protocols in Molecular Biology (Ausubel, et al., 1994).

The mutations were introduced using the Sculptor kit

(Amersham). In the NRSE/RE1 sequence, the mutated sequence was:

B (SEQ ID NO:15) (SEQ ID NO:16)
+24 ACCACTTACA instead of ACCACGGACA, as this mutation was shown to reduce the activity of the NRSE element (Mori et al., 1992).

B In the E-box sequence, the mutated sequence was : -120 TCCTCAGG (SEQ ID NO:17)

B (SEQ ID NO:18)
instead of TCCACTTG. Figure 7 shows that a nuclear protein is able to bind to the wild type sequence, but not to the mutated sequence.

Transfection of Cells.

Neuroblastomas N1E115, human SK-N-Be, HeLa and 3T6 fibroblasts, 293 Human kidney cells and SVLT striatal cells (Evrard et al., 1990) were grown in DMEM + 10% FCS supplemented with 1% glutamine and 1% streptomycin. PC12 cells were grown in DMEM + 10% HS + 5% FCS supplemented with 1% glutamine and 1% streptomycin.

Cells were plated at 10^5 to 4×10^5 cells/60 mm² plates. The next day cells were transfected in 750 μ l of DMEM+

2% Penicillin/Streptomycin for 5 to 12 hours with 1 μ g DNA mixed with 2.5 μ l of Transfectam (IBF/Sepracor) in 150 mM NaCl. The

Luciferase activity was measured 48 hours later. DNA was prepared using Qiagen or Wizard prep (Promega) kits. When plasmid activities were compared, all plasmids were prepared the same day. At least two different DNA preparations were tested for each plasmid. All transfections were done in duplicate and repeated at least three times.

Production of Transgenic Mice.

The luciferase gene from EE1.2-Luci was excised and replaced by the nlsLacZ gene (Kalderon et al., 1984). The β 2-promoter/nlsLacZ fragment was electroeluted from a TAE agarose gel then further purified by ethanol precipitation, and finally resuspended in Tris-HCl 10mM pH 7.5; EDTA 0.1 mM. The DNA solution (3 ng/ml) was injected into fertilized oocytes of C57BL6xSJL hybrids. Staining of tissues was performed as described in Mercer et al., 1991.

See also the methods under figure 8.

Gel Shift Assay

Oligonucleotides were labeled either with γ [32 P]ATP and T4 polynucleotide kinase, or with α [32 P]CTP and Klenow enzyme as in Current Protocols in Molecular Biology. Nuclear extracts were prepared from $\approx 10^7$ cells as described (Bessis et al., 1993). For binding, 1 nmol of labeled oligonucleotide was mixed with 0,5 μ g of protein extract in 10 mM Hepes pH 8, 10% glycerol, 0,1 mM EDTA, 0,1 M NaCl, 2 mM DTT, 0,1 mg/ml BSA, 4 mM $MgCl_2$, 4mM spermidine, 1mM PMSF, 1 μ g polyIdC in 20 μ l. The reaction was incubated for

10 minutes on ice. The DNA-protein complexes were then analyzed on a 7% polyacrylamide gel.

The oligonucleotides used in this experiments were double stranded with the following sequences (the underlined nucleotides are changed between the mutated and the wild type

oligonucleotides):

(SEQ ID NO: 19)
B E-D: 5'-TCCTCCCCTAGTAGTTCCACTTGTGTTCCTAG
(SEQ ID NO: 20)
B Mut-E: 5'-CCTCCCCTAGTAGTTCCTAGGTGTTCCTAGA
(SEQ ID NO: 21)
B S-E: 5'-CTAGCTCCGGGGCGGAGACTCCTCCCC

TAGTAGTTCCACTTGTGTTCCTAG

Results

Characterization of the 5' Flanking Sequences of the Gene Encoding the β 2-subunit

A λ phage containing the gene encoding the β 2-subunit was cloned and a region surrounding the initiator ATG was sequenced (Figure 1). The transcription initiation site was first mapped by RNase protection (Figure 2A). This method allowed us to detect at least three initiation sites. However, minor additional start sites might not have been detected in these experiments. The size of the main protected band was estimated at about 150 nucleotides. To confirm and locate the initiation sites more precisely, we performed both RACE-PCR (Rapid Amplification of cDNA Ends; Frohman et al., 1988) and SLIC (Single Strand Ligation of cDNA; Dumas Milnes Edwards et al., 1991) which consist in the amplification of the primer extension product (Figure 2B). Both techniques allowed us to subclone and sequence the same fragments corresponding to the four initiation sites described in Figure 1. It is probable

that the -13 start site is very rare and was not detected by RNase mapping.

Analysis of the sequence of the flanking region (Figure 1) revealed several consensus DNA binding elements: an Sp1 site (-146), a cAMP responsive element binding (CREB) site (-287; Sassone-Corsi, 1988), a nuclear receptor response element (-344 to -356; Parker, 1993), a GATA-3 site (-1073; Ko and Engel, 1993), and a weakly degenerate Octamer motif (-522). Moreover, an E-box (-118) contained in a dyad symmetrical element could be recognized. The proximal region (-245 to +82) also has an unusually high GC content (67%) and a high number of dinucleotide CpG that may have some regulatory significance (Antequera and Bird, 1993). Finally, a 20 bp sequence identical to the NRSE (Neural Restrictive Silencer Element; Mori et al., 1992) or RE1 (Restrictive Element; Kraner et al., 1992) sequence was found in the 3' end of the 1.2 kbp fragment (+18 to +38).

A 1.2 kbp Fragment of Flanking Sequence of the β 2-subunit Gene Promotes Neuron-Specific Expression in Vitro.

A construct was generated containing the 1163bp EcoRI/Eco47III fragment (from -1125 to + 38) of the β 2-subunit 5' flanking region fused to the Luciferase gene (de Wet et al., 1987) (plasmid EE1.2-Luci). The polyadenylation sites of SV40 were inserted upstream from the β 2-subunit sequences to avoid readthrough. The transcriptional activity of the plasmid EE1.2-Luci was then tested by transient transfection into pheochromocytoma (PC12) cells, neuroblastoma cell lines NIE 115 and SK-N-Be, SVLT, a striatal cell line (Evrard et al., 1990),

NIH3T6 or HeLa fibroblasts and human kidney cell line 293. Using RT-PCR, we verified that the neuroblastomas and the PC12 cells normally express the β 2-subunit mRNA but not the striatal SVLT cell lines or the 3T6 fibroblasts. Figure 3 shows that in PC12 cells and neuroblastomas, the 1.2 kbp fragment is 20 to 180-fold more active in mediating transcription of the reporter gene than in the other cell lines. In fibroblasts, 293 cells and SVLT cells, the transcriptional activity of the 1.2kbp fragment is not significantly higher than that of the promoterless vector (Figure 3). Therefore, the β 2-subunit promoter is not active in these cell lines. These *in vitro* transfection experiments demonstrate that the 1163bp fragment mimics the expression pattern of the endogenous β 2-subunit gene, and thus contains a cell-specific promoter.

The 1163 bp Promoter in Transgenic Mice.

To test the 1163 bp promoter *in vivo*, the EcoRI/Eco47III fragment was linked upstream from the nls- β -galactosidase reporter gene (Kalderon et al., 1984). The polyadenylation signals from SV40 were ligated downstream of the coding sequences. The resulting 4.7 kb fragment was subsequently microinjected into the male pronuclei of fertilized eggs from F1 hybrid mice (C57B16xSJL). DNA extracted from the tails of the offspring was analyzed for the presence of the β -galactosidase gene by the polymerase chain reaction (PCR). Three independent founders were obtained and analyzed for expression.

Two lines (13 and 26) had expression in neurons and the third line did not express at all. This shows that the 1163 bp promoter contains regulatory elements sufficient to drive neuron-specific expression *in vivo*. In the peripheral nervous system PNS, both lines expressed in the same structure. In contrast, in the CNS the labelling pattern of line 26 is a subset of that of line 13. We will only describe line 13 in detail. As expected, most peripheral $\beta 2$ -expressing ganglia expressed β -galactosidase (β -gal), whereas in the CNS only a subset of $\beta 2$ -positive regions expressed the β -gal. For instance, Figure 4C shows that the vast majority of the neurons of the lumbo-sacral spinal cord express the $\beta 2$ -subunit transcripts, whereas only a subset of neurons in the ventral and dorsal horns display β -gal activity.

The expression of the transgene could be detected in the peripheral ganglia in E10.5 and E11 embryos. The labelling was examined in E13 total embryos (Figure 4A) and in brains at later ages (E17, PO and adulthood). At E13, labelling was prominent in PNS: strong labelling was observed in the dorsal root ganglia (DRG, Figures 4 and 5 C, D); some ganglia associated with the cranial nerves (the trigeminal see Figure 5A, geniculate, glossopharyngeal and vagal ganglia); the ganglia of the sympathetic chain (Figure 5C, D); the ganglionic cells of the retina (Figure 5A); and putative parasympathetic ganglia in the cardiac wall (Figure 5B). At E13, clusters of positive cells were also present at several levels of the neuraxis, in both the brainstem and the proencephalon. Clusters of stained neurons were also observed in the ventral and lateral spinal cord.

Later in development (E17), positive neurons were found clustered in several basal telencephalic nuclei whereas dispersed cells were stained in the caudate-putamen. At the diencephalic level, positive clusters were present in the zona incerta and reticular thalamic nucleus, and in many hypothalamic nuclei. In the brainstem, most motor nuclei of cranial nerves (with the exception of the dorsal motor nucleus of the vagus nerve) showed some to high labelling. In addition, the dispersed cells of the V mesencephalic nucleus appeared strongly stained, as well as the pontine nuclei, the prepositus hypoglossal nucleus and a few dispersed cells in the pontine tegmentum.

At P0 in line 13, the distribution of positive cells already appeared more restricted than at previous ages (for example labelling in basal telencephalon and oculomotor nuclei was clearly diminished). In the CNS of adult animals labelled cells were detected only in the hypothalamus. In line 13, some clusters of cells were stained in the mucosa of the gastrointestinal tract (stomach and duodenum) and in the pancreas. Ectopic labelling was detected in the genital tubercle and in several superficial muscles of line 13, but none of these tissues were stained in the line 26.

Identification of a Minimal Cell Specific Promoter

To investigate in more detail the regulatory elements involved in the promoter activity, we generated a series of plasmids containing 5' deletions of the 1163 bp promoter. These plasmids were tested by transient transfection into fibroblasts

and SK-N-Be cells. These two cell lines were chosen as they were the most easily transfected cell lines. Moreover, the neuroblastoma line was initially isolated from peripheral structures (Biedler et al., 1978) and is a convenient tool to study the regulatory elements carried by the 1163 bp promoter.

When 157 bp were deleted from the 5' end of the 1163 bp promoter (plasmid 1006E-Luci, described in Figure 1), the luciferase activity did not significantly change in neuroblastomas but increased in fibroblasts (Figure 6). When 301 bp were further deleted, the activity of the remaining promoter continued to increase in the fibroblasts but not in neuroblastomas (see plasmid 862E-Luci, Figure 6). Thus, the 157 and 301 bp deleted plasmids carry repressor elements which are only active in fibroblasts. However, the truncated 862 bp promoter still displayed a neuron-specific activity (Figure 6, compare activity of 862E-Luci in both cell lines), showing that additional regulatory elements are carried by the 1.2 kbp promoter. Moreover, a repressor could be present between -824 and -245 (compare the activities of 862E and 283E-Luci in the neuroblastomas). This putative regulatory element was not further analyzed. Indeed, a 283 bp promoter (plasmid 283E-Luci) is still ≈ 160 times more active in neuroblastomas than in-fibroblasts, confirming the presence of another neuron-specific regulatory elements in this proximal portion of the promoter.

When 150 bp were deleted from the 5' end of the proximal 283 bp promoter, a very strong decrease of the transcriptional activity was detected in both fibroblasts and neuroblastomas (see

activity of plasmid 133E-Luci). This shows that crucial positive regulatory elements have been deleted. These positive and negative elements were further investigated by deletion and mutation studies of the proximal portion of the promoter.

Negative and Positive Regulatory Elements in the Proximal Region.

The 3' end of the $\beta 2$ -subunit promoter contains putative protein factor binding sites. To analyze the role of these elements in $\beta 2$ -subunit gene regulation, we generated plasmids containing mutations in these binding sites. Using deletion experiments, an activator was detected between -95 and -245 (see Figure 3, the difference between 283E and 133E-Luci). As the E-box located at nt-118 was a good candidate, we analyzed the effect of mutations in this element on transcriptional activity. Table 1A shows a 40% reduction of the transcriptional activity of the mutated promoter compared to that of the wild type promoter. The role of the E-box in non-neuronal tissues was more difficult to assess as the basal level of transcription was already low in fibroblasts.

To further understand the role of the E-Box in the regulation of the promoter, we investigated the protein complexes able to interact with this sequence. Gel shift assays were performed using the 33 bp sequence (nt-135 to -103, oligonucleotide E-D) as a probe. When the 32 -P labelled oligonucleotide was mixed with nuclear extracts from neuroblastomas or fibroblasts, three complexes were observed (Figure 7). All of them were fully displaced by an excess of the unlabelled oligonucleotide E-D. In

contrast, no competition was observed when the competitor oligonucleotide was mutated within the E-Box/Dyad (oligonucleotide Mut.E, see Figure 7 lane "Mut-E"). This shows that the E-box/Dyad is the only element contained within the -135/103 sequence able to bind nuclear protein. This sequence is likely to be involved in the activity of the β -subunit promoter.

An NRSE/RE1 sequence is also present in the proximal region and has been shown to act as a silencer in fibroblasts but not in PC12 cells or neuroblastomas (Kraner et al., 1992; Li et al., 1993; Mori et al., 1992). Point mutation of this sequence in the context of the 1163 bp promoter resulted in a 105-fold increase in the transcriptional activity in fibroblasts, and only a 3-fold increase in neuroblastomas (Table 1A). This sequence is thus responsible for at least part of the cell-specific expression of the $\beta 2$ subunit gene.

Elimination of High Affinity Nicotine Receptor in Transgenic Mice Results in Alteration of Avoidance Learning

The $\beta 2$ -subunit of the nAChR was disrupted in embryonic stem (ES) cells, and mice deficient in this subunit were subsequently generated (Fig. 8). $\beta 2^{-/-}$ -mice were viable, mated normally and showed no obvious physical deficits. Overall brain size and organization were normal (see for example Fig. 9, A and B). Western blot analysis of total brain homogenates using anti- $\beta 2$ monoclonal 270¹¹ (Fig. 8d) and immunocytochemistry throughout the brain using a polyclonal anti- $\beta 2$ antibody⁹ demonstrated that the immunoreactivity detected in control mice was absent in $\beta 2^{-/-}$ -mice and was diminished in $\beta +/ -$ -mice. $\beta 2$ -encoding mRNA was

undetectable in $\beta 2$ -/-mice by *in situ* hybridization using $\beta 2$ -antisense oligonucleotides (Fig. 9A).

The distributions of the $\alpha 4$ - and $\beta 2$ -subunits largely overlap in the brain, and these subunits are thought to combine to form the predominant nAChR isoform in the CNS¹². Based on oocyte expression experiments⁶, $\beta 4$ -is the only subunit identified thus far that might also be able to form functional heteropentamers with the $\alpha 4$ -subunit. The $\beta 4$ -subunit was expressed normally in the brains of $\beta 2$ +/-mice or $\beta 2$ -/-mice, with expression in the medial habenula (MHb) and the interpeduncular nucleus (IPN)¹⁰, and no upregulation elsewhere in the brain to replace the $\beta 2$ -subunit (Fig. 9A). Nor was the expression of the $\alpha 4$ - (Fig. 9A), $\alpha 5$ - or $\beta 3$ -subunit mRNAs significantly altered in mutant mice.

Equilibrium binding experiments have shown that nicotine binds to a population of high affinity sites (KD near 10 nM^{13,14}) whose distribution tallies well with that of the $\alpha 4$ - and $\beta 2$ -subunits¹³⁻¹⁵. Quantitative receptor autoradiography was performed using ³H-nicotine (4 nM) to visualize high affinity nAChR in brain sections from $\beta 2$ +/+, +/- and -/- mice (Fig. 9B). Nicotine binding *in situ* was completely abolished in $\beta 2$ -/- animals, and was reduced by approximately 50% in all brain areas in $\beta 2$ +/-animals implicating the $\beta 2$ -subunit in mediating this high affinity binding.

Electrophysiology of Transgenic Mice.

Neurons of the anterior thalamus, which express very high levels of $\beta 2$ (and $\alpha 4$) subunit mRNAs (Fig. 9A), were studied for an electrophysiological response to nicotine. This area, easily

accessible in a slice preparation, responded consistently to 10 μ M nicotine in wild type animals with an average inward current of 155+/-73 pA which was blocked by 1 μ M dihydro- β -erythroidine. The agonist order of the response was compatible with that seen for α 4/ β 2-containing nicotinic receptors *in vitro*⁶ (nicotine>DMPP>cytisine) (Figure 10A). Anterior thalamic neurons required several minutes to an hour for complete recovery of the agonist response, suggesting that receptor response is prone to desensitization. Moreover, a relatively high dose of 1 μ M was required for a reproducible response, implying that nicotine does not bind to its high affinity site to activate. High affinity nicotine binding sites may therefore be nAChRs in a desensitized conformation.

In β 2-/- mice the response of anterior thalamic neurons to nicotine was completely abolished in 100% of neurons tested (Figure 10B). As a control, neurons in the MHb, where both α 3 and β 4 are strongly expressed, were also tested. Nicotine caused an average inward current of 505 +/- 132 pA in wild type mice, and the agonist potency of this response followed the rank order for the α 3/ β 4 containing receptor (cytisine=nicotine>DMPP) (Figure 10A). As expected, the response of cells in the MHb to nicotine was maintained in mutant mice.

The β 2 subunit is expressed in the ganglia of wild type animals⁸⁻¹⁰, but there was no apparent difference in heart rate or basal body temperature. Spontaneous locomotor activity, which is sensitive to high doses of nicotine and is not modified by drugs

selective for the $\beta 2/\alpha 4$ isoform of the nAChR¹⁶, was not significantly different in $\beta 2/-/-$, $\beta +/1$ and $\beta +/+$ mice.

Cognitive and Behavioral Results.

Learning and memory were examined in mutant and wild type mice using two procedures. The Morris water maze^{17,18} evaluates spatial orientation learning. The performance of mutant mice on this test did not differ from that of wild type mice when tested on the visible platform task, or on the hidden platform task (minimum swim-time reached after 5 days of training: mutants (n=8): 7.4 +/- 1.4 sec; wild type (n=8): 8.2 +/- 2.0 sec). In the transfer test both groups of animals spent approximately 35% of the time in the platform quadrant, with the same number of platform crossings (mutants: 4 +/- 0.4; wild type: 3.9 +/- 0.6).

Retention of an inhibitory avoidance response was assessed using the passive avoidance test, which was also chosen for its pharmacological sensitivity to nicotine administration^{19,20}. This test consisted of a training trial in which the mouse was placed in a well-lighted chamber of a shuttle box, and the latency to enter the adjacent dark chamber was measured. Upon entry to the dark chamber, a mild, inescapable foot shock was delivered, and vehicle or nicotine (10 μ g/kg) was injected into the mouse. Twenty-four (24) hours later, retention was assessed by measuring the latency to enter the dark chamber. Time spent in the light chamber (retention latency) increased proportionally to the applied foot shock in both mutant and wild type mice. However, treatment with nicotine consistently facilitated retention ($p < 0.01$) by shifting the curve upward by approximately 80 sec only

in wild type mice (Fig. 10A). Nicotine administration was completely ineffective in mutant mice. Interestingly, retention latency was significantly higher for mutant mice than for their non-mutant, vehicle-injected siblings ($p < 0.05$) (Fig. 10B).

Increased retention in the passive avoidance test can be observed in animals with a decreased pain threshold or increased emotionality. Therefore, further behavioral testing was performed on all mice included in this experiment. Mutant mice did not differ from their non-mutant siblings for flinch, vocalization or jump response to foot shock. Emotionality was tested by measuring exploratory activity in a two compartment apparatus for 15 min²¹,²². The average time spent in the dark compartment, the locomotor activity in the dark compartment and the transitions between compartments did not differ between the mutant and wild type mice. Therefore, neither changes in pain sensitivity nor changes in emotionality can account for the difference in retention latency observed in passive avoidance testing.

Studies using low doses of nicotine²³ or specific nicotinic agonists¹⁶ suggest that high affinity nAChRs in the brain mediate the effects of nicotine on passive avoidance. Accordingly, nicotine cannot change the performance of $\beta 2^{-/-}$ mice on this test, as they lack high affinity binding sites. The enhanced performance of mutant mice versus wild type mice is quite surprising, however. Several explanations for the paradoxical effect of the $\beta 2$ -subunit mutation can be proposed. One hypothesis is that nicotine injection improves performance of wild type mice on passive avoidance as a result of desensitization, and

thus inactivation of nAChRs, leading to enhanced performance on the test. Therefore, the behavior of mice lacking the receptor might mimic that of mice whose receptors have been desensitized²⁴. Another possibility is that nAChRs may be present in at least two pathways that interact with opposite effects to generate the behavior measured in passive avoidance. If one pathway is physiologically more active than the other, the inactive pathway will be preferentially stimulated by injection of nicotine in wild type animals, while the more active pathway will be preferentially influenced by $\beta 2$ -gene inactivation.

The experiments described above demonstrate that nAChRs containing the β -subunit mediate the effects of nicotine on passive avoidance, a specific learning task. These mice provide a model system for studying the pharmacological effects of nicotine in the CNS, and are useful in elucidating the role of high affinity nAChRs in cognitive processes, nicotine addiction, and dementias involving deficits of the nicotinic system.

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